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PRINCIPAL INVESTIGATOR: Jason Carroll, Ph.D.

CONTRACTING ORGANIZATION: Dana Farber Cancer Institute
                           Boston, Massachusetts 02115-6084

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13. ABSTRACT (Maximum 200 Words)

The mechanisms of estrogen-mediated transcription are not completely understood and as such, the roles of Selective Estrogen Receptor Modulators (SERMs), such as tamoxifen are also poorly understood. Our current work is focused on assessing the relative contributions of the specific promoter sequences within estrogen target genes and how they influence the transcriptional activity by different ligands. Specifically, we have generated breast (MCF-7) and endometrial (ECC1) cancer cell lines with a Lox-Luciferase cassette integrated within the chromatin. These clonal cell lines have been screened by Southern blot and FISH to confirm the presence of a single integration site. Different promoter sequences from estrogen regulated genes have being introduced into these donor cell lines via Cre-mediated recombination. To insert these specific promoter sequences into the same site within the chromatin, we generated an insertion vector containing a multiple cloning sites flanked by Lox sites. After integration of the various promoter sequences we used negative selection to generate cell lines that contained the different promoter sequences within the same chromatin setting. These cell lines are currently being assessed for luciferase activity in the presence of different ligands

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Introduction

The role of estrogen action in driving cancer progression is not completely understood. A large volume of work has focused on determining the role of specific DNA sequences within promoters of target genes using in vitro approaches, which have recently been shown to poorly recapitulate the events that occur in vivo. We have generated breast and endometrial cancer cell lines that contain single entry sites, in the form of a Lox-luciferase integration cassette, that allows us to introduce specific promoter sequences into a chromatin setting to assess transcriptional activity. These clonal cell lines were shown to contain only one integration site and using an entry vector we designed and constructed, we have introduced the promoter sequences of a number of estrogen regulated genes, including pS2, c-Myc, IGF-I and EBAG9 into the same site within chromatin. Initial luciferase assays confirm that the presence of a potent promoter sequence in an in vitro vector does not confer transcriptional activity when present in an in vivo setting. This supports our theory for the requirement of chromatinized sequence to assess the genuine transcriptional activity of a specific fragment of DNA. However, an endometrial cancer cell line with transcriptional activity from the Lox-luciferase cassette has been generated and is being assessed presently. Furthermore, we are re-selecting breast cancer clonal cell lines to identify one with good basal activity for future analysis. This work has clarified our understanding of the importance of chromatin on promoter activity and has provided us with a model to investigate the role of specific promoter sequences in a biologically relevant system.

Body

Specific Aim 1. Assess the specific roles of promoter motifs in regulating transcription of estrogen target genes.

Generation of cell lines

To achieve Specific Aim 1, we were required to generate cell lines derived from MCF-7 breast epithelial cancer cells and ECC1 endometrial cancer cells that contained a Lox-Luciferase construct inserted within the chromatin at one location.

MCF-7 and ECC1 cell lines were already present in the lab. However, we needed to obtain early passage cells for each cell line, since much manipulation was required before the cell line would be usable and we were concerned about the cell lines losing hormone responsiveness. This is a phenomenon that occurs after 35 to 40 passages. Therefore, we obtained early MCF-7 and ECC1 cells and cultured them in growth media.

The Lox-luciferase plasmid that was to be transfected into the cell lines to form an entry site for introduction of promoter sequences was required. We obtained a plasmid containing an hygromycin/gancyclivor selectable marker flanked by two inverted Lox motifs from Eric Bouhassira (Albert Einstein College of Medicine). We modified the vector to contain the luciferase gene with a Kozak sequence 3' to the 3' Lox site.

After sequencing the vector to ensure the modifications were correct and the luciferase insert did not alter the remainder of the plasmid, we transfected the vector into MCF-7 and ECC1 cells. Cells were selected with hygromycin at a concentration determined by does response curves. Antibiotic resistant cells were allowed to grow from single cells into small clonal aggregates of cells, after which two dozen clonal cells from each cell line were selected, trypsinized and plated into individual wells within a 96 well plate.

Each clonal cell line was grown from 96 wells into 6 well dishes and finally into $10cm^2$ culture dishes, after which DNA was collected from each clone. Southern blot analysis was performed on DNA from each clone to establish the number of integrations sites, the presence of full length insert and the absence of concatamers of vector insert. An example of the data is shown in Figure 1.

We selected two MCF-7 and two ECC1 cell lines that met the criteria of containing a single full length, intact Lox-Luciferase insert, without concatamers and performed Fluorescent In Situ Hybridization (FISH) on these cell lines. FISH analysis allowed us to confirm that these cell lines did in fact contain only one integration site and provided information about the location of the site on a chromosome level. Figure 2 shows one MCF-7 and one ECC1 example of this.

Construction of Insertion vector

To insert specific promoter sequences into the established cell lines, we needed to construct an insertion vector which would contain: 1) a multicloning site (MCS) to allow introduction of various promoter sequences, 2) inverted Lox elements flanking the MCS and other elements to allow for Cre-mediated recombination between the promoter sequence in the insertion vector and the Lox cassette within the chromatin, 3) Gal4 binding domain repeats to allow for potential immunoprecipitation using an antibody to Gal4 after overexpression of Gal4 protein in the cell line. Using standard cloning techniques we produced a vector that contained all of these elements. The vector was called pJay-1. pJay-1 was sequenced to ensure it contained the correct sequence.

Production of pJay-1 promoter vectors and recombination in cells

The promoter sequences (1kb regions) of pS2, IGF-I, c-Myc and EBAG9 were PCR amplified from MCF-7 total genomic DNA and cloned into the MCS of pJay-1. These were all sequenced. Empty pJay-1 and pJay-1 containing the four promoter sequences were transfected into the MCF-7 and ECC1 cell lines using Lipofectamine 2000. Cre

expressing vector (a kind gift from Eric Bouhassira, Albert Einstein College of Medicine) was co-transfected to induce recombination between the promoter sequences flanked by the inverted Lox sites and the Lox sites within the chromatin of the cell lines. Cells were grown for three days, after which negative selection was induced using gancyclivor. Gancyclivor was used at a concentration previously determined by dose response curves. Cells were selected for two weeks after which surviving cells were expanded to 10cm^2 dishes. DNA was collected from each cell line and assessed for the integration of the specific promoter sequences into the chromatin. This was achieved by performing PCR using a reverse primer within the luciferase gene that had been integrated into the chromatin and a specific forward primer against each of the promoter sequences that were recombined into the Lox cassette (see Figure 3).

Transcriptional activity from promoter sequences

To assess the estrogen induced activity of pS2, EBAG9, c-Myc and IGF-I promoter sequences (as measured by the ability to drive luciferase transcription) in a chromatin context, we deprived each of the promoter containing cell lines of hormones for three days and added back either vehicle or estrogen for 24 hours. Protein lysate was collected and assayed for luciferase activity. However, no luciferase activity was detectable in either the MCF-7 or ECC1 promoter containing cell lines. To identify possible reasons why these promoter sequences, which have previously been shown to contain potent transcriptional activity in *in vitro* systems, could not activate transcription, we performed Chromatin Immunoprecipitation (ChIP) of ER followed by PCR of the various promoter sequences. We could not find ER association with any of the promoter sequences suggesting that the integration site of the Lox-Luciferase cassette was not conducive to euchromatic conditions because ER could not associate with these regions of the chromatin. To further investigate whether transcriptional activity from the integration sites was possible, we constructed a pJay-1 insertional vector containing the potent CMV promoter. This vector was transfected into MCF-7 and ECC1 Lox cell lines and antibiotic selection was applied. Assessment of luciferase activity suggested that the MCF-7 cell line had no transcriptional activity while the ECC1 cell line had low activity.

Future plans

Despite that fact that the ECC1 Lox cell line contained some luciferase transcriptional activity when a CMV promoter was placed upstream in a genuine chromatin context, the small range of activity suggests that these cell lines will not be applicable for the assessment of weaker promoter sequences. However, it does confirm that a cancer cell line can be generated to contain one single entry point within chromatin and that specific fragments of DNA can be inserted into the same region with relative ease.

We are currently reselecting cell lines to use as host cells for assessment of promoter activity, but we have cloned the GFP gene into the Lox-Luciferase vector and have transfected this into MCF-7 and ECC1 cell lines. This will allow us to FACS sort cells that contain high GFP activity which will either be an indicator of multiple integration sites, or integration sites in euchromatic regions of chromatin. Using Southern blot analysis we should be able to identity clonal cell lines that have only one integration site, but within a region of the genome that is susceptible to transcriptional activity. Once this is established, we can proceed with the next stage of the project, although we have the constructs made and the reagents optimized. Furthermore, we know that we can successfully introduce various promoters into the same site in a cell line.

Figure 1

Southern blot analysis of two MCF-7 clonal cell lines (clones 9 and 10) after digestion with either AlwNI and PciI (lanes 1) or XbaI (lanes 2) restriction enzymes. PciI will digest the vector in the luciferase gene, whereas AlwNI does not cut anywhere in the vector. As such, AlwNI/PciI digestion will produce products of various sizes, depending on the distance between the integration site and the closest AlwNI restriction site in the chromatin. This allows us to assess the number of integration events. Restriction digestion with XbaI allows us to assess the presence of concatamers of vectors. Both MCF-7 clones 9 and 10 contain a single integration site and do not contain concatamers. The 7.1kb marker represented the expected minimum size of the insert, supporting the conclusion that the insert is full length

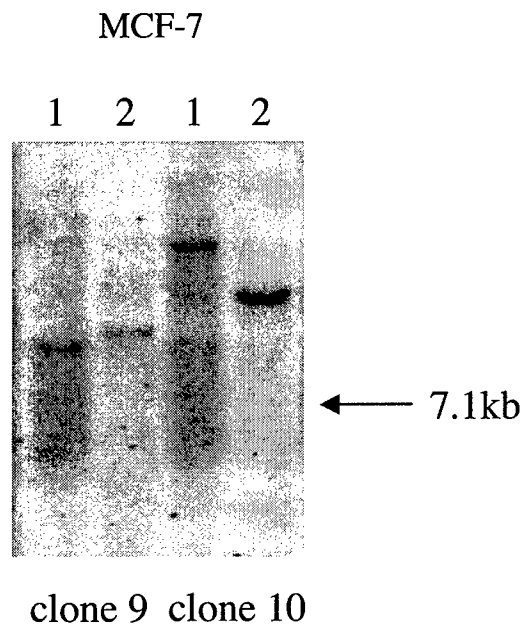

MCF-7

1   2   1   2

◄——— 7.1kb

clone 9 clone 10

Figure 2

FISH analysis of Lox-Luciferase integration number and site in MCF-7 and ECC1 clonal cell lines. Cells from MCF-7 clone 9 and ECC1 clone 23 were subjected to metaphase spread and FISH analysis was performed using a probe against the Lox-hygromycin-Luciferase cassette. As can be seen in both cell lines, integration occurred only once and in both cases, appears to be distant from the centromere, which can potentially provide transcriptional restrictive conditions.
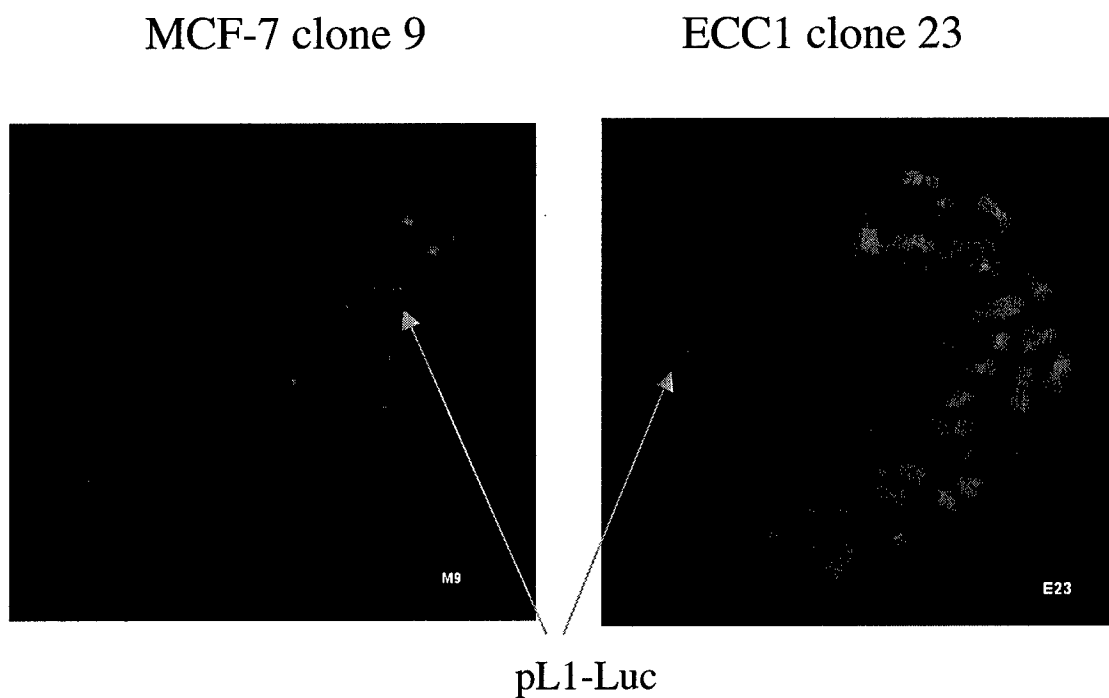

MCF-7 clone 9   ECC1 clone 23

pL1-Luc

Figure 3

Assessment of promoter integration into the MCF-7 clone 9 cell line. After pJay-1 promoter transfection in the presence of Cre recombinase and subsequent gancyclivor selection, DNA was extracted and PCR amplification was performed. A common luciferase reverse primer was used, but a unique promoter specific primer was used with DNA from the appropriate cell line. As can be seen, all four promoter sequences (pS2, c-Myc, IGF-I and EBAG9) could be successfully integrated into the same site within the chromatin of the MCF-7 clone 9 cell line.

MCF-7 clone 9

Primer pair   Myc   IGF-I   pS2   EBAG9   pJay1
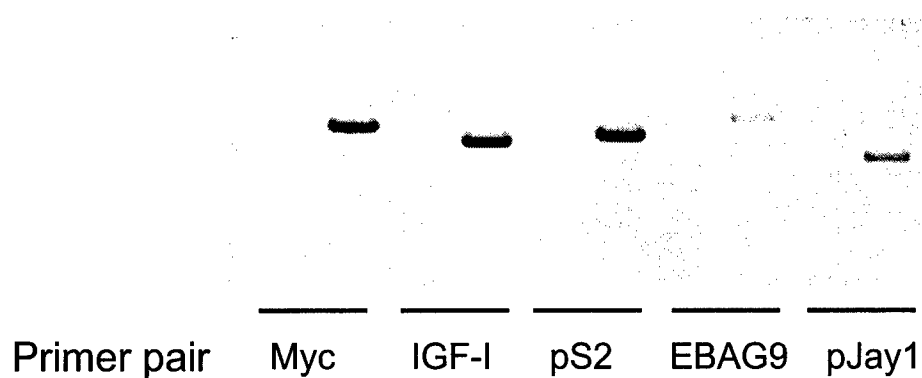
Key Research accomplishments

- Establishment of cell lines that contain one single integration site

- Use of different approaches to prove that these cell lines contain a full length, functional insertion site

- Production of pJay-1 insertional vector for multiple uses

- Construction of pJay-1 vectors containing the promoter sequences of four known estrogen regulated genes

- Identification of the Cre-mediated recombination of promoter sequences into the chromatin of a cell line

- Establishment of a system that for the first time, allows us to introduce interesting fragments of DNA into a chromatin setting and allows us to introduce DNA into the same region of chromatin for assessment of transcriptional activity

Reportable outcomes

1- Abstract and poster presented at Keystone 2004 Nuclear Receptor meeting

2- Seminar presented at Project Program Grant meeting at Harvard Medical School (including Weinberg, Brugge, Livingston, LaBaer, Sicinski groups)

3- Data shown at 2004 Annual Harvard symposium (poster)

4- Development of breast and endometrial cancer cell lines for investigation of promoter activity in a genuine in vivo setting

5- Construction of a number of vectors including a common pJay-1 insertional vector

Conclusions

We have undertaken a detailed project that will potentially allow us to investigate the promoter activities and protein recruitment in an in vivo setting. A significant volume of work has focused on the essential elements within the promoter sequences of estrogen target genes (Dubik and Shiu, 1992; Petz et al., 2002; Porter et al., 1996; Teng et al., 1992; Umayahara et al., 1994; Vyhlidal et al., 2000; Weisz and Rosales, 1990). These studies have shown that the transcriptional activity of the 1kb promoter proximal regions requires Sp-1, AP-1 and other motifs. However, recent work has shown that chromatinized DNA functions very differently from in vitro transfected DNA. Therefore, we sought to produce a system whereby we could assess the transcriptional activity of promoter regions in a chromatinized setting. Furthermore, we wanted to be able to mutant specific regions and re-introduce the promoter into the same setting and assess the changes in transcriptional activity that particular mutations have. Ultimately, we aim to be able to correlate promoter activity with protein recruitment by performing indirect ChIP using an antibody against Gal4, which has the ability to bind to Gal4 motifs adjacent to our transfected promoter sequence. To this end, we have constructed breast and endometrial cancer cell lines that have a single point of entry Lox-luciferase cassette inserted in the chromatin. We have successfully produced an insertion vector and have used this vector to introduce the promoters of c-Myc, IGF-I, pS2 and EBAG9 into the same region within chromatin. However, the transcriptional activity of the luciferase gene is relatively low, even when driven by a potent promoter. As such, we are re-selecting cell lines that contain integration sites with greater transcriptional potential, after which we will re-introduce the promoter regions and assess their transcriptional activity.

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